

# Cytokine-Induced Nitric Oxide Production Inhibits Mitochondrial Energy Production and Impairs Contractile Function in Rat Cardiac Myocytes

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- OBJECTIVES** The present study examined whether nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) can directly inhibit aerobic energy metabolism and impair cell function in interleukin (IL)-1 $\beta$ -stimulated cardiac myocytes.
- BACKGROUND** Recent reports have indicated that excessive production of NO induced by cytokines can disrupt cellular energy balance through the inhibition of mitochondrial respiration in a variety of cells. However, it is still largely uncertain whether the NO-induced energy depletion affects myocardial contractility.
- METHODS** Primary cultures of rat neonatal cardiac myocytes were prepared, and NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> (NOx) in the culture media was measured using Griess reagent.
- RESULTS** Treatment with IL-1 $\beta$  (10 ng/ml) increased myocyte production of NOx in a time-dependent manner. The myocytes showed a concomitant significant increase in glucose consumption, a marked increase in lactate production, and a significant decrease in cellular ATP (adenosine 5'-triphosphate). These metabolic changes were blocked by co-incubation with N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis. Sodium nitroprusside (SNP), a NO donor, induced similar metabolic changes in a dose-dependent manner, but 8-bromo-cyclic guanosine 3',5'-monophosphate (8-bromo-cGMP), a cGMP donor, had no effect on these parameters. The activities of the mitochondrial iron-sulfur enzymes, NADH-CoQ-reductase and succinate-CoQ-reductase, but not oligomycin-sensitive ATPase, were significantly inhibited in the IL-1 $\beta$  or SNP-treated myocytes. Both IL-1 $\beta$  and SNP significantly elevated maximum diastolic potential, reduced peak calcium current (I<sub>Ca</sub>), and lowered contractility in the myocytes. KT5823, an inhibitor of cGMP-dependent protein kinase, did not block the electrophysiological and contractility effects.
- CONCLUSIONS** These data suggest that IL-1 $\beta$ -induced NO production in cardiac myocytes lowers energy production and myocardial contractility through a direct attack on the mitochondria, rather than through cGMP-mediated pathways. (J Am Coll Cardiol 2000;35:1338–46) © 2000 by the American College of Cardiology

Endogenously or exogenously produced nitric oxide (NO) can cause negative inotropic effects in the cardiovascular system (1–5). Consequently, a great deal of attention is being focused currently on conditions that expose the heart to elevated concentrations of NO. In examining the causes

of inflammation-induced myocardial injury, it has been observed recently that the proinflammatory cytokines interferon (IFN)- $\gamma$ , interleukin (IL)-1 $\beta$ , and tumor necrosis factor (TNF)- $\alpha$  can increase NO synthesis by elevating the expression of inducible nitric oxide synthase (iNOS) (6), causing a sustained depression in myocardial contractility (3,4,7), as well as protracted hypotension during septic shock (8). However, despite this recent elucidation of the conditions that promote NO production in the inflamed heart, mechanisms of the NO-induced injurious effects remain poorly understood.

Two potential mechanisms of NO damage are currently favored. The first invokes an indirect mode of injury in

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Manuscript received August 6, 1998; revised manuscript received October 27, 1999, accepted December 15, 1999.

#### Abbreviations and Acronyms

|              |  |
|--------------|--|
| ATP          | = adenosine 5'-triphosphate                    |
| 8-bromo-cGMP | = 8-bromo-cyclic guanosine 3',5'-monophosphate |
| IL-1 $\beta$ | = interleukin-1 $\beta$                        |
| iNOS         | = inducible nitric oxide synthase              |
| L-NMMA       | = N <sup>G</sup> -monomethyl-L-arginine        |
| NO           | = nitric oxide                                 |
| SNP          | = sodium nitroprusside                         |

which the NO-induced activation of soluble guanylate cyclase causes the increased production of cGMP, which then decreases myocardial contractility by decreasing cytoplasmic Ca<sup>2+</sup> concentration (1–4). The second and more direct mechanism proposes that NO may inhibit the aerobic energy-producing processes in the heart by directly altering mitochondrial activity. Recent reports have shown that iNOS-produced NO can cause inhibition of mitochondrial respiration in a variety of cells, resulting in disturbances in energy balance and cytotoxicity (9,10). Significantly, a similar ability of cytokine-stimulated NO production to inhibit mitochondrial activity has been documented recently in cardiac myocytes as well. These studies (11,12) suggested that iNOS-produced NO can diminish mitochondrial activity, but they left unanswered the question of whether the NO-induced energy depletion affects myocardial contractility.

The purpose of the present study was therefore to elucidate the relationship between NO-induced energy depletion and cell dysfunction and to determine which of the two potential mechanisms for the NO-associated injury occurs in IL-1 $\beta$ -treated cardiac myocytes. Using a previously established model of IL-1 $\beta$ -mediated iNOS induction in cardiac myocytes (13), we examined the ability of NO to disrupt the glycolytic and energy-producing pathways by monitoring not only glucose and lactate content in the culture media, but also lactate and ATP (adenosine 5'-triphosphate) content in the myocytes themselves. To examine the possible involvement of indirect injury by cGMP, we treated myocytes with 8-bromo-cyclic guanosine 3',5'-monophosphate (8-bromo-cGMP), a cGMP analogue. To test for direct inhibition of mitochondrial activity, we assayed the enzymatic activity of the NO-susceptible mitochondrial iron-sulfur enzymes, NADH-coenzyme Q (CoQ) reductase (complex I), succinate-CoQ reductase (complex II), and oligomycin-sensitive ATPase (complex V). Furthermore, we measured membrane potential, calcium current, and myocyte contractility to assess myocyte function.

## METHODS

**Materials.** Recombinant IL-1 $\beta$  (lymphocyte-activating factor,  $2 \times 10^7$  U/mg protein) was a generous gift from Otsuka Pharmaceutical Co. The 8-bromo-cyclic guanosine

3',5'-monophosphate (8-bromo-cGMP), gentamycin, and collagenase were obtained from Sigma Chemical, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) from Calbiochem, and other chemicals from Nacalai Tesque.

**Culture of neonatal rat cardiac myocytes.** Primary cultures of rat neonatal cardiac myocytes were prepared as previously described with some modifications (14). Briefly, cardiac ventricles from one- or two-day-old Wistar rats were minced and dissociated with 0.2% collagenase (Sigma Type I). The dispersed cells were incubated in 100-mm culture dishes (Falcon) for 60 min at 37°C, and the nonattached cardiac myocytes were collected and seeded into 100-mm culture dishes ( $8 \times 10^6$  cells per dish). The myocytes were incubated in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Bioserum Lenexa, Kansas). Bromodeoxyuridine (BrdU, 100  $\mu$ mol/liter) was added during the first 48 h to prevent proliferation of nonmyocytes.

At the end of the BrdU treatment, we routinely obtained spontaneously beating myocyte-rich cultures (final cell density,  $10^5$  cells/cm<sup>2</sup>) with 90% to 95% myocytes, as assessed by immunocytochemical test for myosin heavy chain (15). The myocytes were then incubated for 36 to 48 h in DMEM containing 0.5% FBS without BrdU, followed by a final incubation in 10 ml fresh DMEM containing 0.5% FBS. During this final incubation, cardiac myocytes were treated with one of the following: 1)  $10^{-4}$  mol/ml or  $10^{-3}$  mol/liter sodium nitroprusside (SNP); 2)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter 8-bromo-cGMP; 3) 10 ng/ml IL-1 $\beta$  in the presence or absence of 3 mmol/liter L-NMMA; 4) 10 ng/ml IL-1 $\beta$  in the presence or absence of  $5 \times 10^{-4}$  mol/liter aminoguanidine; 5) 10 ng/ml IL-1 $\beta$  in the presence of  $10^{-7}$  mol/liter KT5823; or 6)  $10^{-3}$  mol/liter 8-bromo-cGMP in the presence of  $10^{-7}$  mol/liter KT5823. Unless indicated otherwise, treatment was continued for 48 h at which time ATP and mitochondrial enzyme activity were determined in the myocytes, and NOx (NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>), glucose, and lactate concentrations were measured in the culture media.

**NOx assay.** The NOx (nitrite and nitrate) concentrations in the culture media were determined in an autoanalyzer (ENO-10; Eicom, Kyoto, Japan). Samples were applied to an analytical column combined with a copperized cadmium reduction column to reduce NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup>, which was then reacted with Griess reagent to produce a product absorbing at 540 nm (16). Authentic NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were used as reference standards.

**Glucose and lactate analysis.** Glucose concentration was measured enzymatically using the Detaminder GL-E kit (Kyowa Medics, Tokyo, Japan). Aliquots of culture media were added to a phosphate-buffered reaction mix containing 2.4 IU/ml ascorbate oxidase, 0.7 mmol/liter sodium *N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HSDA),

2 IU/ml pyranose oxidase, 3.4 IU/ml peroxidase, and 0.4 mmol/liter 4-aminoantipyrine, at pH 6.5. Glucose content was determined spectrophotometrically at 37°C by measuring quinone absorbance at 585 nm.

Lactate content was measured enzymatically, using the Detaminer LA kit (Kyowa Medics, Tokyo, Japan). Aliquots of culture media were added to a phosphate-buffered reaction mix containing 0.9 mmol/liter *N*-ethyl-*N*-(3-methylphenyl)-*N'*-acethylethylenediamine (EMAE), 0.7 IU/ml lactate oxidase, 3.4 IU/ml peroxidase, and 0.4 mmol/liter 4-aminoantipyrine, at pH 6.25. Lactate content was determined spectrophotometrically at 37°C by measuring quinone absorbance at 555 nm.

**Measurement of ATP content.** Cultured cardiac myocytes ( $8 \times 10^6$  cells) were treated with 500  $\mu$ l 0.6N ice-cold perchloric acid and centrifuged at 3,000g for 5 min at 4°C. The supernatants were neutralized with KOH to pH 5.0 to 7.0. After 10 min the extracts were centrifuged at 13,000g for 5 min at 4°C to remove the KClO<sub>4</sub>, and the ATP in the supernatants was measured by high-performance liquid chromatography (HPLC: LC-9A liquid chromatograph, Shimadzu, Kyoto, Japan) with a column of STR ODS-M (Shimadzu) (17).

**Mitochondrial isolation.** Mitochondria were isolated according to the method of Sordahl et al. (18), with some modifications. Briefly, each culture dish ( $8 \times 10^6$  cells) was washed with ice-cold phosphate buffered saline (PBS) containing 15 mmol/liter Na<sub>2</sub>HPO<sub>4</sub>, 5 mmol/liter NaH<sub>2</sub>PO<sub>4</sub>, 145 mmol/liter NaCl, at pH 7.3. The myocytes were collected by scraping and were washed with ice-cold buffer containing 25 mmol/liter K<sub>2</sub>HPO<sub>4</sub>, 25 mmol/liter KH<sub>2</sub>PO<sub>4</sub>, and 1 mmol/liter EDTA, at pH 7.2. Pooled myocytes (approximately  $8 \times 10^7$  cells) were homogenized in 1.6 ml of potassium-phosphate buffer containing 0.5 mmol/liter phenylmethanesulfonyl fluoride (PMSF) with a Polytron PT 10-35 tissue processor (Kinematica Instrument, Switzerland) three times at a setting of 7 for 10 s, and the homogenate was centrifuged at 27,000g for 10 min. The supernatant was discarded and the pellet was suspended in 5 ml isolation medium consisting of 0.18 mol/liter KCl, 5 mmol/liter Tris-HCl, and 10 mmol/liter EGTA, at pH 7.2. The suspension was centrifuged at 1,000g for 10 min, and the supernatant was collected and centrifuged at 12,000g for 10 min. The mitochondrial pellet was washed once and resuspended in 400  $\mu$ l low-salt medium consisting of 20 mmol/liter HEPES, 1.0 mmol/liter MgCl<sub>2</sub>, and 2.0 mmol/liter EGTA, at pH 7.0. The protein concentration in the mitochondrial suspension was determined by the Lowry method (19).

**Measurement of mitochondrial enzyme activity.** Mitochondria were sonicated, and NADH-CoQ reductase activity was assayed spectrophotometrically at 37°C by measuring the initial rate of NADH oxidation at 340 nm in the presence or absence of rotenone (15  $\mu$ mol/liter), according

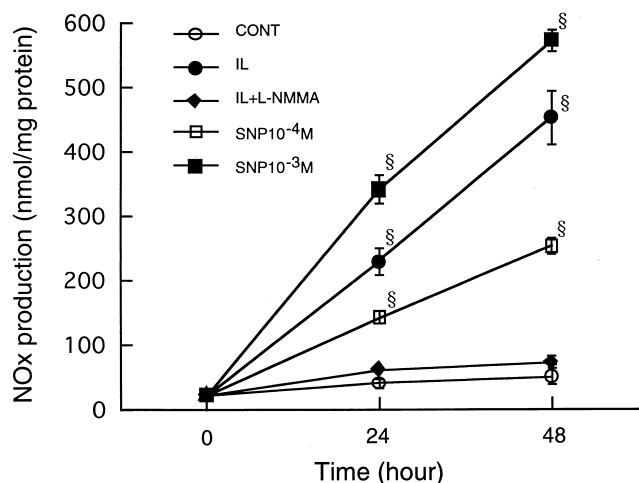
to the method of Hatefi and Rieske (20). Mitochondrial succinate-CoQ reductase activity was assayed spectrophotometrically at 37°C by measuring the reduction of 2,6-dichloroindophenol sodium (DCIP) at 600 nm, according to the method of Ziegler and Rieske (21). Mitochondrial ATPase activity was measured spectrophotometrically by coupling the reaction to a pyruvate kinase and lactate dehydrogenase system, according to the method of Lowe (22). Mitochondrial ATPase activity was determined at 37°C by monitoring the decline of NADH oxidation at 340 nm in the presence or absence of oligomycin (10 ng/ml).

**Electrophysiology.** A whole-cell voltage-clamp method was applied to the isolated neonatal cells to measure calcium current (*I*<sub>Ca</sub>) (23). Current clamp mode was used in the whole-cell configuration when membrane potential was recorded. An Axopatch 200A amplifier (Axon Instrument, Foster City, California) was used for this purpose. All data were digitized by DigiData 1200 A/D converter (Axon Instrument). Data acquisition and commands were controlled with pClamp software (version 6.0; Axon Instrument). Electrodes were prepared from glass capillaries with filament (outer diameter 1.2 mm; inner diameter 1.0 mm, Narishige, Tokyo, Japan), and filled with a solution containing 1) current clamp mode: 120 mmol/liter KCl, 10 mmol/liter NaCl, 1 mmol/liter MgCl<sub>2</sub>, and 20 mmol/liter HEPES, pH 7.3, adjusted with KOH; 2) voltage clamp mode: 125 mmol/liter L-aspartic acid, 25 mmol/liter tetraethyl-ammonium chloride, 5 mmol/liter Na<sub>2</sub>ATP, 1 mmol/liter MgCl<sub>2</sub>, 1 mmol/liter EGTA, and 5 mmol/liter HEPES, pH 7.3, adjusted with CsOH, whose final concentration was 160 mmol/liter.

Membrane potential was recorded continuously in current clamp mode while cells were superfused with a modified Tyrode solution whose composition was as follows: 135 mmol/liter NaCl, 5.4 mmol/liter KCl, 1.8 mmol/liter CaCl<sub>2</sub>, 1 mmol/liter MgCl<sub>2</sub>, 0.33 mmol/liter NaH<sub>2</sub>PO<sub>4</sub>, 10 mmol/liter HEPES, 10 mmol/liter glucose, pH 7.4, adjusted with NaOH. The *I*<sub>Ca</sub> was recorded every 10 s in voltage clamp mode. The KCl in superfusing solution was replaced with 20 mmol/liter CsCl<sub>2</sub> in this mode. Membrane voltage was held at -80 mV for 9.5 s, stepped up to -40 mV for 0.25 s to inactivate sodium current, and then jumped to 0 mV for 0.25 s to evoke *I*<sub>Ca</sub>. Peak *I*<sub>Ca</sub> was measured relative to the zero-current level.

**Contractility.** Nomarski differential interference-contrast microscopy with enclosed heated chamber (Nikon DIAPHOT-TMD300) was used for visualization of cardiac myocytes. A series of images of cardiac myocytes during a contraction was recorded on the charge-coupled device (CCD; Nikon Instech/SONY LVR-3000AN-NS). Cell length was determined directly from the images using an edge-detection system.





**Figure 1.** Time course of  $\text{NO}_2^-/\text{NO}_3^-$  ( $\text{NO}_x$ ) production. Cardiac myocytes were incubated with either (a)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter sodium nitroprusside (SNP), or (b) 10 ng/ml IL-1 $\beta$  (IL) in the presence or absence of 3 mmol/liter  $\text{N}^G$ -monomethyl-L-arginine (L-NMMA) for the indicated time, and  $\text{NO}_x$  production in the culture media was measured as described in Methods. Control preparations contained no additives. The interaction between five groups and time course was significant ( $p < 0.0001$ ).  $\$p < 0.0001$  vs. control ( $n = 6$ ).

**Statistical analysis.** All experiments were repeated at least six times. The data in Figures 1 and 2 were analyzed by two-way analysis of variance (ANOVA), and the data at each time point were analyzed by one-way ANOVA and Bonferroni's multiple comparison. The data in Figures 3–8 were also analyzed by one-way ANOVA and Bonferroni's multiple comparison. Data are expressed as mean  $\pm$  SE, and a  $p$ -value of less than 0.05 was considered to be statistically significant.

## RESULTS

**Cell death in cultured cardiac myocytes.** Following our previously described histochemical methods (24) we employed combined staining with Hoechst 33258, desmin, and in-situ end-labeling (ISEL) of fragmented DNA to monitor apoptosis of the isolated cardiomyocytes (24). We did not observe apoptosis in cells grown in 0.5% FBS for up to 48 h with or without IL-1 $\beta$  treatment (data not shown). We also did not detect a significant increase in creatine kinase activity in the media, or trypan blue uptake by the treated cells, thus ruling out an increase in necrotic cell death (data not shown).

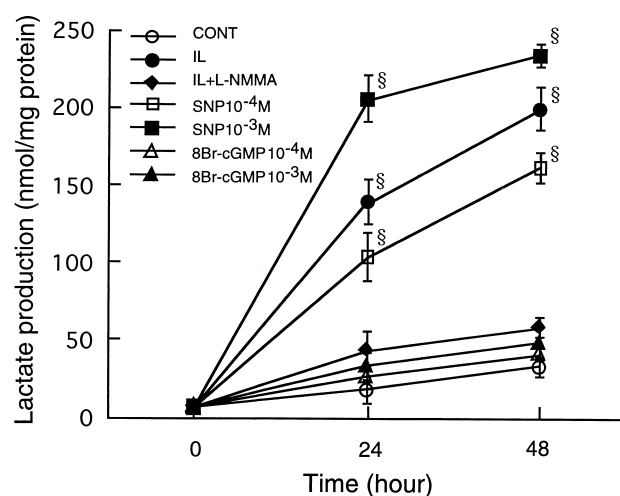
**Induction of  $\text{NO}_x$  production by IL-1 $\beta$ .** Because NO is rapidly oxidized to more stable products such as nitrite and nitrate, NO production in the cultured myocytes was evaluated by measuring  $\text{NO}_x$  in the culture media. IL-1 $\beta$  (0 to 100 ng/ml) increased  $\text{NO}_x$  production in a dose-dependent fashion, reaching a near-maximum of  $225 \pm 20$  nmol/mg protein at 10 ng IL-1 $\beta$ /ml after 24 h (data not

shown). We therefore used this concentration of IL-1 $\beta$  for the subsequent experiments.

The time-dependent kinetics of  $\text{NO}_x$  production are shown in Figure 1. Treatment of myocytes with 10 ng/ml IL-1 $\beta$  for 48 h caused a marked linear accumulation of  $\text{NO}_x$  in the culture media to levels approximately 11-fold greater than control. The IL-1 $\beta$ -induced  $\text{NO}_x$  production was completely inhibited by co-incubation with 3 mmol/liter L-NMMA, an inhibitor of NO synthesis. Prolonged incubation with SNP, an exogenous NO donor, also increased  $\text{NO}_x$  concentration in a dose- and time-dependent manner, with  $10^{-4}$  mol/liter and  $10^{-3}$  mol/liter SNP increasing  $\text{NO}_x$  levels by 5- and 14-fold over control, respectively, after 48 h.

**Lactate accumulation.** Interleukin-1 $\beta$  increased lactate concentration in the culture media in a time-dependent manner (Fig. 2). Treatment with IL-1 $\beta$  for 48 h resulted in a marked accumulation of lactate in the culture media to levels approximately four-fold greater than control. Lactate production was significantly inhibited by 3 mmol/liter L-NMMA. Also, SNP significantly increased lactate production in a dose-dependent manner. With both IL-1 $\beta$  and SNP, lactate production declined after 24 h. In contrast, treatment with 8-bromo-cGMP, an exogenous cGMP donor, did not significantly increase lactate accumulation.

**Glucose consumption.** The glucose consumption rate was calculated from the difference between the concentration of glucose in the culture media before, and 24 h after, incubation (Fig. 3). Treatment of myocytes with IL-1 $\beta$

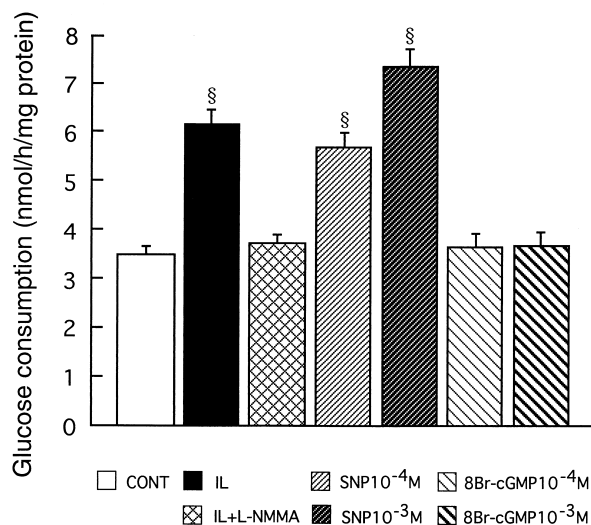


**Figure 2.** Time course of lactate production. Cardiac myocytes were incubated with (a)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter sodium nitroprusside (SNP), (b)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter 8-bromo cGMP, or (c) 10 ng/ml IL-1 $\beta$  (IL) in the presence or absence of 3 mmol/liter  $\text{N}^G$ -monomethyl-L-arginine (L-NMMA) for the indicated time, and lactate production was measured in the media as described in Methods. Control preparations contained no additives. The interaction between seven groups and time course was significant ( $p < 0.0001$ ).  $\$p < 0.0001$  vs. control ( $n = 6$ ).

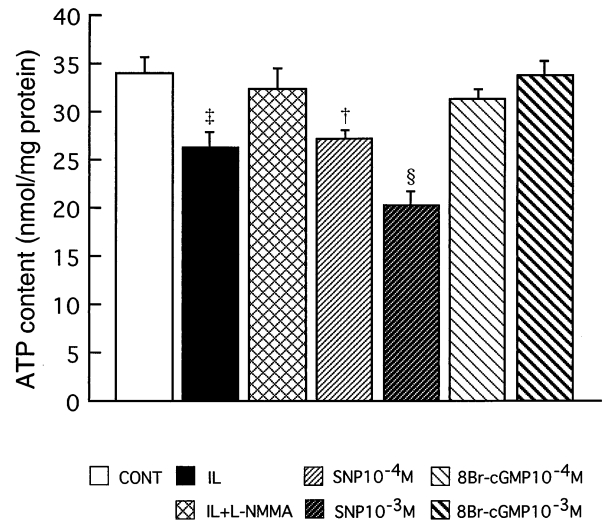
increased glucose consumption by almost two-fold over control. This increase in glucose consumption was completely abolished by L-NMMA. The SNP also significantly increased glucose consumption. In contrast, 8-bromo-cGMP did not affect the glucose consumption rate in the cardiac myocytes. Glucose consumption in myocytes treated with either IL-1 $\beta$  or SNP declined significantly by 48 h (data not shown).

**ATP content.** The ATP content in the control cultured myocytes did not change for up to 48 h of incubation. However, ATP concentration decreased to 78% of control in myocytes treated with IL-1 $\beta$  after 48 h (Fig. 4). This decline in ATP content was completely blocked by 3 mmol/liter L-NMMA. Also, SNP decreased ATP content significantly in a dose-dependent manner. In contrast, 8-bromo-cGMP did not alter ATP content. KCN ( $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter), a mitochondrial inhibitor, significantly decreased ATP content in a dose-dependent manner in cultured myocytes. However, the decline in ATP content induced by KCN alone was significantly smaller than that caused by SNP. Moreover, when added to myocytes treated with IL-1 $\beta$  or SNP, KCN significantly exacerbated the inhibition of ATP production, further lowering ATP content by an additional 10% after 48 h (data not shown).

**Mitochondrial enzyme activities.** Treatment of cultured myocytes with IL-1 $\beta$  for 24 h significantly lowered the activities of mitochondrial NADH-CoQ reductase and



**Figure 3.** Glucose consumption rate in cardiac myocytes. Myocytes were incubated with (a)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter sodium nitroprusside (SNP), (b)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter 8-bromo cGMP, or (c) 10 ng/ml IL-1 $\beta$  (IL) in the presence or absence of 3 mmol/liter N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) for 24 h, and glucose consumption rate was measured as described in Methods. Control preparations received no additives.  $\S p < 0.0001$  vs. control (n = 6).

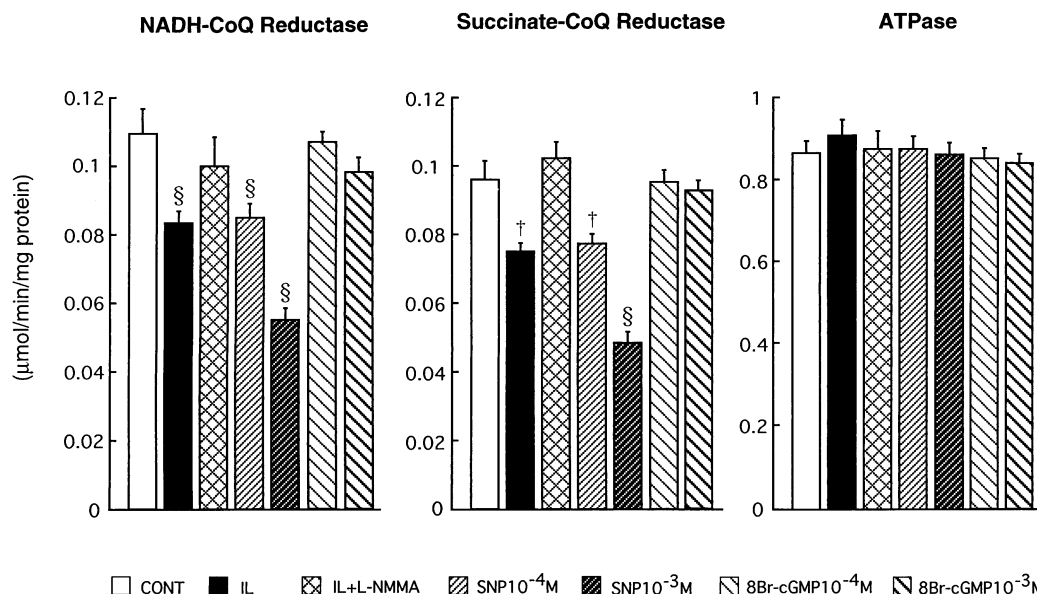


**Figure 4.** ATP content in cardiac myocytes. Myocytes were incubated with (a)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter sodium nitroprusside (SNP), (b)  $10^{-4}$  mol/liter or  $10^{-3}$  mol/liter 8-bromo cGMP, or (c) 10 ng/ml interleukin (IL)-1 $\beta$  in the presence or absence of 3 mmol/liter N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) for 48 h, and ATP content was measured as described in Methods. Control preparations received no additives. <sup>†</sup> $p < 0.01$ ; <sup>‡</sup> $p < 0.001$ ; <sup>§</sup> $p < 0.0001$  vs. control (n = 6).

succinate-CoQ reductase to 76% and 78% of control, respectively (Fig. 5). Continuing IL-1 $\beta$  treatment for 48 h further exacerbated this inactivation (data not shown). The L-NMMA completely abolished the IL-1 $\beta$ -induced suppression of these activities. Moreover, SNP also significantly decreased the activities of these enzyme in a dose-dependent manner. The activity of oligomycin-sensitive ATPase did not change with IL-1 $\beta$  or SNP. The activities of these enzymes were not affected by 8-bromo-cGMP.

**Electrophysiological effects.** Maximum diastolic potential was  $-54 \pm 2.8$  mV in control cells (n = 16),  $-32 \pm 3.4$  mV in IL-1 $\beta$ -treated cells (n = 19,  $p < 0.001$  vs. control), and  $-25 \pm 3.0$  mV in SNP  $10^{-3}$  mol/liter-treated cells (n = 7,  $p < 0.001$  vs. control) (Fig. 6). Action potential duration fluctuated with the maximum diastolic potential, and neither IL-1 $\beta$  nor SNP significantly affected this duration. The  $I_{Ca}$  was evoked at a membrane potential of 0 mV. Peak  $I_{Ca}$  was  $584 \pm 89$  pA in control cells but was significantly lowered with IL-1 $\beta$ , SNP, or 8-bromo-cGMP (Fig. 7). The IL-1 $\beta$ -induced decline in peak  $I_{Ca}$  was significantly blocked by aminoguanidine or L-NMMA. KT5823, an inhibitor of cGMP-dependent protein kinase, did not significantly affect the IL-1 $\beta$ -induced reduction in  $I_{Ca}$ , although it almost completely restored the 8-bromo-cGMP-induced loss of  $I_{Ca}$ .

**Contractility.** Treatment of myocytes with either SNP or IL-1 $\beta$  significantly decreased contractility after 48 h (Fig. 8). This IL-1 $\beta$ -induced decrease in contractility was completely blocked by aminoguanidine or L-NMMA. The



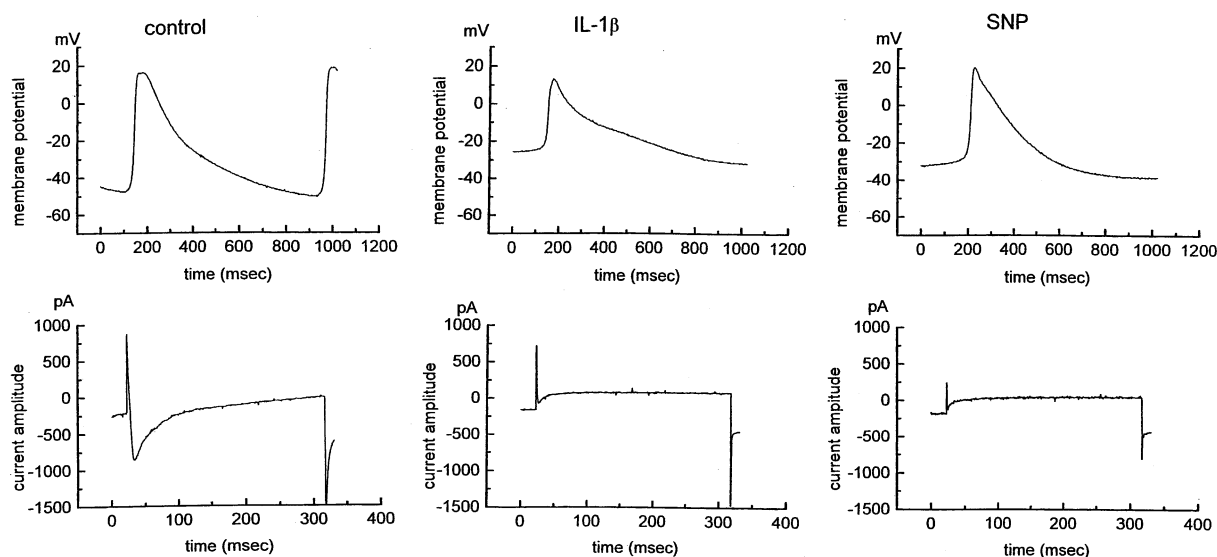
**Figure 5.** Mitochondrial enzyme activity in cardiac myocytes. Myocytes were incubated in the presence of 10 ng/ml IL-1 $\beta$  (IL), with or without 3 mmol/liter N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), and 10<sup>-4</sup> mol/liter or 10<sup>-3</sup> mol/liter sodium nitroprusside (SNP). The activity of the indicated mitochondrial enzymes was assayed as described in Methods. Control preparations received no additives. All activities are in units of  $\mu\text{mol/min/mg protein}$ .  $^{\dagger}p < 0.01$ ;  $^{\S}p < 0.0001$  vs. control ( $n = 6$ ).

addition of KT5823 did not restore contractile function in IL-1 $\beta$ -treated myocytes.

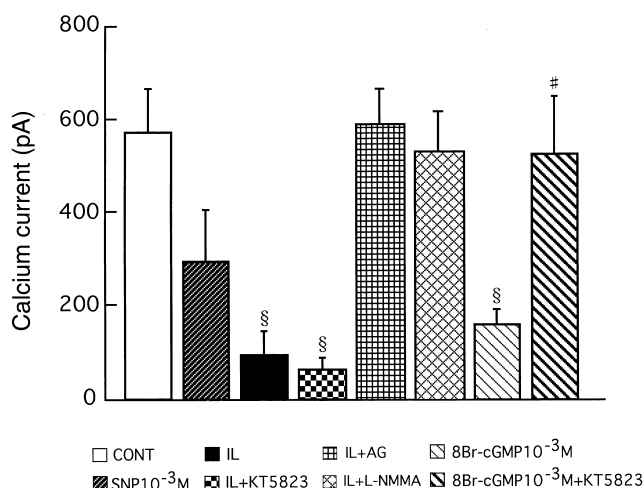
## DISCUSSION

This study shows for the first time that cytokine-induced NO production in neonatal cardiac myocytes causes the attenuation of cellular energy production as a result of the direct inhibition of mitochondrial enzymes. This inhibition was associated with

the disruption of the electrophysiological properties of these cells, and an attendant contractile dysfunction. We show that treatment of myocytes with the proinflammatory cytokine IL-1 $\beta$  causes the inhibition of mitochondrial respiration, an increase in anaerobic glycolysis and lactate production, and a resultant decline in ATP. Therefore, the principal significance of our data is that the data provide a direct molecular link between the overproduction of NO and the physiological manifestations of the resultant injury in inflamed myocardium.



**Figure 6.** Electrophysiological effects in cardiac myocytes. Representative traces show membrane potential and calcium current ( $I_{Ca}$ ) in myocytes treated with 10 ng/ml IL-1 $\beta$  (IL) or 10<sup>-3</sup> mol/liter sodium nitroprusside (SNP) as described in Methods.



**Figure 7.** Peak calcium current in cardiac myocytes. The cells were incubated with 10 ng/ml IL-1 $\beta$  (IL) in the presence or absence of 10<sup>-7</sup> mol/liter KT5823, 5  $\times$  10<sup>-4</sup> mol/liter aminoguanidine (AG), and 3 mmol/liter N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), 10<sup>-3</sup> mol/liter 8-bromo cGMP in the presence or absence of 10<sup>-7</sup> mol/liter KT5823, or 10<sup>-3</sup> mol/liter sodium nitroprusside (SNP) for 48 h, and calcium current (I<sub>Ca</sub>) was measured as described in Methods. §p < 0.0001 vs. control; #p < 0.01 vs. 8-bromo-cGMP (n = 13).

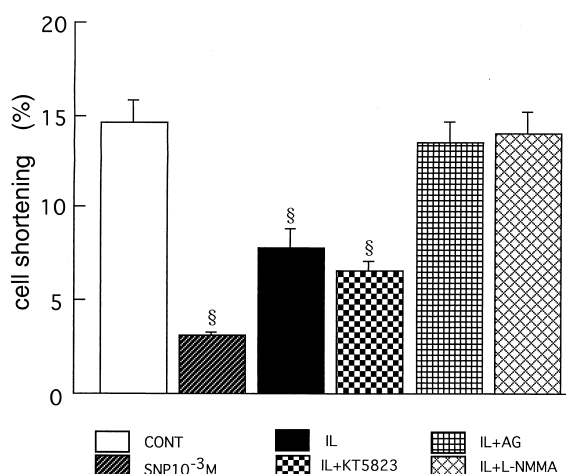
Three lines of evidence confirm that NO was indeed the effector molecule for these changes. First, SNP, a NO donor, mimicked these injurious effects. The fact that cyanide alone did not cause similar effects rules out the possibility that the effect of SNP was caused by the release of CN<sup>-</sup>. Second, L-NMMA, a competitive antagonist of NOS, completely blocked the detrimental effects of IL-1 $\beta$ . Third, aminoguanidine, a selective iNOS inhibitor, blocked the NO-induced electrophysiological effects. Our data, therefore, strongly suggest that the overproduction of NO by iNOS can cause direct injury to the mitochondrial respiratory machinery, inhibit energy production, and cause contractile dysfunction. The fact that the cGMP analogue, 8-bromo-cGMP, did not have any deleterious effects on mitochondrial activity, ATP content, glucose consumption, or lactate production indicates that the effect of NO was direct and was not mediated through the activation of soluble guanylate cyclase.

**NO and mitochondrial respiration.** It is presently well-established that IL-1 $\beta$  can induce the expression of iNOS in cardiac myocytes (13), resulting in a burst of NO production capable of causing negative inotropic, or outright cytotoxic, effects (3-5). Recent studies have also shown that cytokine-induced NO production can inhibit mitochondrial activity in isolated cardiac myocytes (11,12). Moreover, other studies, measuring tissue O<sub>2</sub> consumption, have shown that NO release from vascular endothelial cells appears to play an important physiological role in the regulation of mitochondrial respiration in skeletal muscle (25). However, our study is the first to establish that

NO-induced mitochondrial dysfunction causes myocardial energy depletion in cardiomyocytes, and we demonstrate a link between this energy reduction and contractile dysfunction.

In addition, it is noteworthy that most of the previous studies in this field incubated the myocytes in serum-deprived medium for prolonged periods of time. These culture conditions may have resulted in significant apoptosis (26), thereby confounding the cytokine effects. In contrast, the present study was carried out in medium containing 0.5% FBS, which did not cause apoptosis in our hands either with or without IL-1 $\beta$  treatment (data not shown).

The specific targets of NO in the mitochondria remain to be fully elucidated. Recently, NO was shown to cause the inhibition of mitochondrial respiration in macrophages (27), as well as in cultured smooth muscle cells, by inhibiting complex I and II activity (9). The inhibition of cytochrome oxidase was also demonstrated in isolated cardiac myocytes exposed to a combination of IL-1 $\beta$  and INF- $\gamma$  (12). Our present study sheds additional light on this issue by demonstrating that both endogenous (IL-1 $\beta$  induced) and exogenous (SNP induced) NO inhibits the mitochondrial enzymes NADH-CoQ reductase and succinate-CoQ reductase—metalloproteins containing iron-sulfur clusters known to be highly sensitive to direct oxidative modification by the free radical NO (28). Previous studies have demonstrated that NO can react with the metal clusters in these enzymes to form injurious nitrosyl complexes (29,30). Our data suggest that the injurious effects of NO are probably oxidative in nature. It is not clear at this time why the



**Figure 8.** Myocyte contractility. The cells were incubated with 10 ng/ml IL-1 $\beta$  (IL) in the presence or absence of 10<sup>-7</sup> mol/liter KT5823, 5  $\times$  10<sup>-4</sup> mol/liter aminoguanidine (AG), and 3 mmol/liter N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), or 10<sup>-3</sup> mol/liter sodium nitroprusside (SNP) for 48 h, and cell shortening was measured as described in Methods. §p < 0.0001 vs. control (n = 10).



oligomycin-sensitive ATPase was not inhibited by our experimental conditions.

**NO and energy metabolism.** Inhibition of the respiratory enzymes by NO in our study dictates that the rate of anaerobic glycolysis increases in the myocytes to compensate for the reduced energy supply. Such increased activity of the glycolytic pathway would rapidly increase glucose consumption and lactate production. The present study did, in fact, document these characteristic changes in both the IL-1 $\beta$ -treated and SNP-treated myocytes. This effect was totally inhibited by L-NMMA, but was not mimicked by the addition of exogenous 8-bromo-cGMP, confirming that the inhibitory action of NO was not mediated through intracellular signaling by NO-inducible soluble guanylate cyclase (31).

In view of its lower energy-yielding potential, anaerobic glycolysis would not be expected to maintain normal ATP content in the actively beating myocytes. This, and the increased acidotic conditions known to accompany anaerobic glycolysis and lactate production, would presumably cause the ultimate inhibition of myocyte contractility (32–34). We did, in fact, observe ATP depletion, electrophysiological alterations, and contractile dysfunction in IL-1 $\beta$ -treated myocytes. The decline in ATP content, which reached approximately 25% after 48 h of IL-1 $\beta$  treatment, was inhibited by L-NMMA. Exogenous SNP also produced a substantial decrease in ATP content, providing support for the direct involvement of NO. Moreover, the addition of the respiratory inhibitor cyanide to the IL-1 $\beta$ -treated cells significantly exacerbated ATP depletion, confirming that, in our model, ATP depletion was dependent on the severity of inhibition of glycolysis and mitochondrial respiration.

**Energy depletion and cell dysfunction.** Reduced energy production in mitochondria in the presence of IL-1 $\beta$  should result in impairment of cell function. Our data show elevated maximum diastolic potential, reduced peak  $I_{Ca}$ , and reduced contractility in IL-1 $\beta$ - or SNP-treated myocytes. This loss in contractility is presumably attributable to the inactivation of the calcium current and lower calcium entry (35,36). KT5823, a selective inhibitor of cGMP-dependent protein kinase (37), did not significantly restore the IL-1 $\beta$ -induced reduction of  $I_{Ca}$ , but it did reverse the effects of 8-bromo-cGMP, confirming that the NO-induced ATP depletion directly alters intrinsic cellular activity, independently of cGMP-associated signaling pathways. Thus, our data strongly suggest that direct inactivation of the mitochondrial energy-producing apparatus by NO, and the resultant disruption of calcium regulation, are both the likely cause of the cessation of contractile activity.

However, at this time we cannot exclude the possibility that direct inactivation of calcium-regulating membrane proteins may also contribute to this effect. Our studies demonstrated a decline in ATP of only 25%, which may not be sufficient to account for the cessation of contractility. In

fact, the possibility of modulation of the calcium-regulatory apparatus by NO in a similar model was raised recently by Bick et al. (38), who showed that treatment of myocytes with a number of cytokines, including IL-1 $\beta$ , for 2 or 18 h resulted in increased calcium transients and contractility, suggesting that NO may directly affect sarcolemmal function. We are unable to explain this apparent discrepancy between our two conflicting sets of data. However, in their conclusions, Bick et al. emphasize the time-dependent nature of their observed effects. The apparent conflict may therefore reflect the much longer incubation time that we employed.

**Conclusions.** In summary, therefore, the present study shows that NO production in cardiac myocytes in response to the inflammatory mediator IL-1 $\beta$  causes the inactivation of selected oxidant-sensitive mitochondrial enzymes, resulting in the inhibition of mitochondrial respiration, energy depletion, electrophysiological dysfunction, and strong inhibition of contractility. Because we did not detect a significant increase in apoptosis or necrosis, our observations are unlikely to be attributable to nonspecific toxicity by NO. Our data suggest that the inhibition of respiration is not caused by means of cGMP, but is attributable in large measure to the direct oxidative modification by NO of mitochondrial enzymes.

We cannot discount, at this time, the possibility that the production of O<sub>2</sub><sup>-</sup> and/or H<sub>2</sub>O<sub>2</sub> by iNOS (39,40), or the production of peroxynitrite from NO and O<sub>2</sub><sup>-</sup> (41), may have contributed to this oxidative stress. Nor can we exclude the possibility that this injury is compounded by multiple other known effects of NO such as the auto-ADP-ribosylation of glycolytic enzymes (42), inhibition of ribonucleotide reductase (43), activation of poly (ADP-ribose) synthetase (44), inhibition of the citric acid enzyme aconitase (45), or others. However, the present study provides strong evidence for the production of excessive and injurious amounts of NO during cardiac inflammatory reactions that can have significant direct detrimental effects on cardiac respiration and contractility. Knowledge that these detrimental effects of NO may be caused largely by direct oxidation of mitochondrial enzymes may prove useful in designing protective therapies for inflammation-induced myocardial injury.

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